Immunofluorescence in Diagnostic Virology

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Since the introduction of tissue culture methods for the growth and recognition of viruses in the 1940s, most viral diagnosis that depends on the detection of virus rather than measurement of antibody has used this sensitive and versatile, but expensive and cumbersome technique. It is ironic, therefore, that recognition of viral antigens in clinical specimens was first introduced long before the development of this tissue culture technology. In the 1920s infectious disease clinicians and pathologists were taking the lymph of smallpox lesions and showing that one could agglutinate it with specific antisera. In the 1930s Parker and Muckenfuss first developed the complement fixation reaction using lymph mixed with antibody and a fresh source of complement.⁵ In fact, this became the procedure of choice for the laboratory diagnosis of smallpox before and even after the general use of tissue culture for viral isolation.

Immunofluorescence (IF) was also recognized early as a potentially valuable tool for detection of viral antigens,^{3,7,8} but its use as a procedure that could supplement and, at times, replace tissue culture in diagnostic laboratories has been only gradually gaining acceptance. IF has, of course, been used at several stages in the diagnosis of viral diseases: the measurement of antibody, particularly when there is some highly specific antigen in question, as with Epstein-Barr virus or cytomegalovirus; the identification of viruses in tissue culture; and the direct detection of viruses in body fluids. It is this last application that I will elaborate on in this paper.

The detection of viruses by IF in specimens obtained directly from the patient takes advantage of two features of the method that are inherent in IF but are not widely recognized. First, the sensitivity of IF is enormously increased by the capacity (indeed, the necessity) to detect intracellular antigens. This natural concentration of antigens in specific compartments allows the detection of much smaller quantities than would be otherwise possible. Second, there is an enormous increase in the specificity of the method contributed by the morphologic features of the fluorescence produced by each specific virus in each specific sort of specimen. Not only can non-specific fluorescence, an unfortunate accompaniment of the IF technique with almost any tissue examined, be rapidly assessed and ignored; but staining of the structures other than the ones particularly sought (for example, bacteria) can be quickly discerned and appropriately discounted.

The rapidity of the technique is another advantage. Preparation of the specimens is crucial to the success of the method; it usually requires only a few minutes, and staining, particularly by the direct method, can be accomplished in an hour or two. Most solid-phase antigen detection systems require longer incubation periods than this. A second advantage, to be illustrated below, is that the antigens of clinical specimens are often more stable than their infectivity, and this allows the use of the method under circumstances where transportation of specimens is slow or difficult. And finally, because the antigens observed by IF are usually intracellular, and therefore in an immunologically protected position, they can often be detected even in the presence of

antibody. This means that IF often remains positive later in the course of disease than in either culture² or tests for soluble antigen.⁴

In spite of these advantages, there are still many viral diagnostic laboratories that do not use IF for the detection of antigens in body fluids. There are several reasons for this, all of which should, with time, be overcome. First, skill and experience are necessary in obtaining of suitable specimens from patients, in the preparation of these specimens for staining, and in the examination and reading of the slides by the microscopist. Second, excellent reagents are absolutely essential to achieve the sensitivity and specificity that are possible and necessary to render the method competitive with culture techniques. Such reagents are now available in Europe and available to a limited extent in the United States or other countries in this hemisphere. The first of these problems, that of experience and training, should gradually be overcome as viral diagnostic tests are more widely used and the demand for rapid diagnosis increases. However, there are obstacles to this that will be discussed later in this paper. Reagents should become available both as the requirements are more widely recognized by the industries involved and as monoclonal antibodies for each of the important virus groups are made and produced by commercial firms. Some of the problems faced by those trying to adapt monoclonal antibodies to uses in solid-phase systems are fortunately not encountered in IF. Avidity, although important, is not such a crucial problem as it is when picogram quantities of soluble antigens are being measured. Sandwich methods are most efficient when two monoclonal antibodies recognizing two different determinants are used. This is not necessary for IF, since only a single antiviral antibody is used. If a wider spectrum of antibodies is needed, it may ultimately be possible to mix several monoclonal antibodies and achieve relatively quickly the spectrum desired.

Some time and effort should be spent in discussion of the proper method for obtaining specimens for IF diagnosis in human disease. Although this seems a trivial subject, an understanding of the techniques involved and the potential pitfalls is absolutely essential for success in diagnosis.

Since whole cells are needed, only certain specimens are adequate. For those viruses shed from the respiratory tract (a large proportion of the viruses of man), the chances of recovering whole cells shed from the respiratory epithelium should be maximized. Although swabs have been used with success, a far more reliable source of cells is a sample of respiratory secretion.⁷ This is most easily obtained from the nasopharynx. Although tracheal epithelium may localize the virus more certainly in the lower respiratory tract, this is usually not necessary, and, indeed, the effort to obtain the specimen in infants and children often yields only a sample of esophageal epithelium (sometimes mixed with gastric secretion) and causes at the same time unnecessary discomfort. A No. 8 French plastic feeding tube is connected to a thumb valve, a trap, and then a source of gentle mechanical suction. The tube is introduced along the floor of the nose until it reaches the nasopharynx and is then slowly withdrawn as suction is applied. The resultant sample of secretion, which is usually rich in shed epithelial cells and virus, is then transported on ice to the laboratory where it is mixed with about 10 ml of buffered saline and centrifuged to deposit the cells. All steps are performed gently since the infected cells are fragile and easily disrupted. The supernatant is removed and the cell button again mixed with buffered saline. This process is repeated until there is no mucus found (two or three times). The cells are then deposited in 8 mm square spots on a clean microscope slide, allowed to dry in air, and fixed for ten minutes in acetone at 4°C. Some skill is required both in separating mucus from cells and in judging how many cells are required to make a slide that will render an accurate positive or negative diagnosis.

This method is applicable to the diagnosis of infections by influenza viruses,

parainfluenza viruses, respiratory syncytial virus, adenovirus, measles, rubella virus, and coronaviruses. If antisera were available, the method might be applicable to enteroviruses and rhinoviruses as well, but the multiple serotypes involved have thus far impeded success with these groups. The choice of which antiserum to use in the laboratory requires a knowledge of the seasonal epidemiology of each of the viruses, some information about the clinical presentation of the patient sampled, and enough competence in the details of infectious disease to match intelligently the patient and the potential infecting viruses. In most instances several viruses should be tested for, if only as a source of controls for that particular sample. The proper and practical use of controls will be discussed more fully below.

For those viruses found in vesicular lesions on the skin, namely herpes simplex and varicella, the lesion should be gently scraped with a scalpel and the cells obtained should be deposited in two or more drops of saline on the surface of a clean slide.¹ These will usually be fairly large chunks, which should immediately be broken up as finely as possible with two #26 needles or, preferably, dissecting needles. The slides are then allowed to dry, fixed as above, and stained appropriately.

The other major source of cells for IF is biopsy or autopsy. It has rarely been valuable to search for viral antigens in leukocytes, either in the peripheral blood, cerebrospinal fluid, or serosal fluids. Autopsy or biopsy specimens may be examined either in frozen sections or in touch preparations. The latter are often easy to prepare, of good quality for IF, and in some instances superior to frozen sections since they tend to be thin and well dispersed, and to have less non-specific staining.

Any good fluorescence microscope may be used for IF viral diagnosis, but the reading of multiple specimens is facilitated by the use of incident light and a 40 $\times$ oil-immersion objective. The latter we have found to be essentially the only objective we need in clinical work. The medium magnifying power is adequate for observation of cellular detail, an absolute necessity for accuracy, and yet the field is large enough and the amount of light sufficient for rapid scanning of an entire specimen. Oil immersion, while not routinely used at this power, reduces the light scattering produced when specimens are examined directly and obviates the need for a coverslip. We find that glycerol, either buffered or used untreated from the container, has adequate optical properties and is much less irritating to mucous membranes than high performance immersion oils.

While the proper use of multiple controls is and should be the rule with fluorescence in the research laboratory, in the diagnostic setting the rigorous insertion of positives, negatives, preimmune sera, conjugate controls, blocking, and so on would become unnecessarily cumbersome. We depend heavily on the examination of large numbers of specimens, the repetitive use of the same sera and conjugates, and the accumulated experience of the observer. Moreover, replicate slides must always be made from each specimen, so that in situations where there is any question the required controls can be done, or the staining procedure can be repeated.

In general, before they are used in the clinical setting, all reagents should have been titrated in tissue culture, tested on frozen clinical slides of known positives and known negatives, shown to exhibit no cross-reactions with other species of virus, and examined with regard to non-specific reactions in multiple clinical specimens. Few antisera are perfect, and imperfect sera can be used with great accuracy if their characteristic imperfections are well known. Each clinical specimen should be judged as to its adequacy and rejected if there are not enough cells present or if the level of non-specific fluorescence is too high and irreducible. Finally, to make a positive reading on a given slide, certain criteria must be absolutely insisted on. The fluorescence must be in the proper cell type (i.e. not a squamous epithelial cell if respiratory viruses are being

sought) and must be in the appropriate part of the cell for that virus (i.e. not in the nucleus for a parainfluenza virus). The morphology of the fluorescence must be right for that particular virus. Respiratory syncytial virus produces fine punctate fluorescence often denser around the limiting membrane of the cell, with the occasional bright spot of a cytoplasmic inclusion body. Herpes virus looks entirely different, often staining the nucleus or the entire cell densely with little morphology visible. The fluorescence must, as every microscopist knows, be of exactly the right color: if fluorescein is used, it must be apple green, not any other green or yellow. In virtually every case, at least two positive cells must be seen. Usually if two positive cells are found there are many other *almost* positive cells around, and one can often find material that looks as though a positive cell had been disrupted either during the obtaining or preparation of the specimen. A blatantly positive cell as an isolated finding in a blatantly negative background may mean that that cell was inadvertently transferred to that slide by means of a pipet tip from another slide.

Whenever the microscopist is dealing with an even slightly unfamiliar situation, controls should be used. Positive and negative tissue culture controls should be stained. There should be a negative tissue control (e.g. a negative brain frozen section if virus is being sought in brain). The suspect specimen should be stained for several viruses, and if possible a preinoculation serum should be used for the particular virus most suspected. If the conjugate being used in an indirect staining procedure is in the least unfamiliar, the antiviral serum should be omitted as a conjugate control. Finally, if there is still doubt, or if the finding is unusual or unexpected, blocking tests should be done. The most important ingredient for specificity is, in the long run, however, the experience of the microscopist. There will always be judgement involved in the use of IF for viral diagnosis, and many years of experience and of comparison of culture and IF results are essential for the accurate reading of difficult slides.

How can a technician gain the confidence necessary to call a specimen positive or negative when tissue culture results either disagree or are unavailable? This is probably the most vexing problem in the use of immunofluorescence in a routine viral diagnostic laboratory, and undoubtedly the one which will continue for some time to inhibit its widespread application. There is unfortunately no substitute for experience: the repetitive comparison of IF results with culture in innumerable different samples, with constant application of irreproachable standards of honesty and quality control. Alternatively, the administrative framework can be made available for repetitive comparisons of readings with those in a laboratory where experience with the technique has been extensive. This type of system has been used with respiratory viruses in England,⁶ and is used for rabies diagnosis in this country. For this reason, good, versatile IF microscopists are usually trained in tissue culture diagnostic laboratories. If they leave virus isolation behind, they must know and admit their limitations, and periodic return to comparative situations (either with culture or with more experienced readers) would seem to be an important part of the maintenance of high standards.

When all aspects of technique are satisfactorily cared for, the results of IF diagnosis come exceedingly close to those of tissue culture. In some situations they are better. One of these with which we have had recent experience is the testing of specimens for labile viruses, which are sent by taxi or bus from outlying hospitals. Our record in the past two years illustrates this well. In 1980–1981, during the respiratory syncytial virus (RSV) season, 90% of positive specimens came from patients within our own hospital. In that year, we recovered 94% of the total positive specimens in tissue culture, and detected 88% by IF. In 1981–1982, 46% of RSV-positive specimens were sent to us from other hospitals in Boston and the suburbs of Boston. In that season the

sensitivity of culture fell to 75% while that of IF rose to 90%. Overall, specimens coming from outside the hospital were found to be IF-positive but culture-negative four times as often (36%) as those coming from within the hospital (9%).

Thus, now in our laboratory IF of clinical specimens has become a routine adjunct to our diagnostic armamentarium. We use it occasionally as the only diagnostic method, omitting culture: when specimens are old; when an unexpected load on the laboratory exhausts our supply of tissue culture; or sometimes in epidemiologic studies where a single virus is being sought during a known outbreak. It serves the irreplaceable function of giving the physician and patient an early answer, often allowing for timely withdrawal of antibiotics or intelligent infection control procedures. It has become a test much in demand by our medical staff and, by making virus recognition immediately available, has brought the laboratory in all its functions closer to the patient and the physician.

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